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(54) Title: HISTIDINE KINASE ASSAY		
(57) Abstract The present invention is directed at methods and kits for assaying histidine kinases, particularly fungal and bacterial 2-component histidine kinases. Additionally, the invention discloses methods for using the histidine kinase assay in in whole-cell and in vitro assays as a tool for screening putative inhibitors of histidine kinase to identify an entire new class of fungal and bacterial inhibitors.		

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HISTIDINE KINASE ASSAY

BACKGROUND OF THE INVENTION

Fungi are an extremely large group, with about 250,000
5 species widely distributed in essentially every ecosystem.
Humans are exposed to fungi from the moment of birth.
Fortunately, only 200 or so species are pathogenic, although
many nonpathogenic fungi cause allergy symptoms. The
majority of fungal exposures and infections are self-
10 limiting in intact hosts. However, in patients with a
compromised immune system, infections, even by fungal
organisms with low virulence, can be life threatening; for
example, fungal infections of leukemia patients account for
50% of fatalities. Nosocomial bloodstream infections have a
15 similar fatality rate.

During the last two decades, the incidence of fungal
infections, especially involving immunocompromised patients,
has dramatically increased. This is due, in part, to the
tremendous advances in medicine that permit the saving of
20 patients with neoplastic and immunocompromising diseases
that would otherwise not have survived. It is ironic that
many of these triumphs to modern medicine succumb to fungal
infections for which there are few or no drugs available for
treatment.

25 Host defenses against fungal infections include intact
epithelial surfaces, the mucociliary lining of the
respiratory tract, neutrophils, macrophages and lymphocytes.
Factors that negatively affect any of these defenses
predispose patients to fungal infections. The main risk
30 factors include corticosteroid treatment, antibiotics,

diabetes, lesions of the dermis or epidermis, malnutrition, neutropenia, chemotherapy, surgery and indwelling catheters.

Fungal infections have been classified into two types: superficial and deep (or systemic). Implicated fungi have been classified by two schemes: One scheme is to classify by cellular morphology (yeasts, filamentous fungi and dimorphic fungi), and the other scheme is to classify fungi as "true pathogens" or "opportunistic pathogens." The most important fungal pathogens, judged by incidence, are *Candida* spp., especially *albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii*.

Treatment of deep or systemic infections is difficult due to the lack of effective and safe antifungal antibiotics. Even after 29 years of use, Amphotericin B (a polyene) is still the drug of choice to treat systemic fungal infections. The apparent mode of action of Amphotericin B (AmB) is to complex with membrane sterols, resulting in membrane distortion and leakage of intracellular contents. However, the utility of AmB is limited due to its high toxicity to human cells and because AmB therapy is fraught with side effects, including: renal dysfunction, fever, chills, hypotension and even cardiac failure. These shortcomings underscore the clear need for new antifungals. A major hindrance to the discovery of such compounds is the lack of simple assay systems for screening potential inhibitors to different fungal-specific enzymes.

Of the antifungal agents other than AmB which are capable of treating systemic infections, most inhibit enzymes that catalyze key reactions in the biosynthesis of polymeric compounds that comprise fungal cell walls. These enzymes are attractive targets given that fungal and human cells differ fundamentally in that fungal cells are encased

in a cell wall that protects the cells from an osmotically and immunologically hostile external environment, whereas human cells lack such a cell wall.

Other enzymatic activities are crucial to proper cell-wall formation besides those involved in the synthesis of cell-wall polymers, including enzymes involved in the so called 2-component signal transduction pathways. Unlike the enzymes involved in cell-wall polymer biosynthesis, however, these enzymes have not been tested as targets for fungal

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SUMMARY OF THE INVENTION

The current invention describes a method for assaying the enzymatic activity of kinases. In particular, the invention describes a method for assaying histidine kinase activity, and more specifically, 2-component histidine kinase activity in a broad range of fungi, for example *Candida albicans*, *Neurospora crassa* and *Aspergillus fumigatus*, as well as bacteria. The invention further discloses how the assay can be used as a screening tool for identifying fungal and bacterial inhibitors which would have utility as antifungal and antibacterial agents. Still a further aspect of the invention is a description of peptides which can be used as substrates in kinase assays, particularly 2-component histidine kinase assays.

More specifically, one aspect of this invention is the identification of two genes *os-1* and *cos-1* (*Candida osmotic sensitive*), that exist in diverse fungi. The proteins encoded by *os-1* and *cos-1*, *oslp* (SEQ ID NO: 1) and *coslp* (SEQ ID NO: 2 represents about two-thirds of the entire sequence), respectively, are homologous to *slnlp*, the yeast osmosensing 2-component histidine kinase, and certain bacterial 2-component histidine kinases. Importantly,

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humans lack cognates of either of these genes.

Consequently, the inventors have identified the proteins encoded by these genes as attractive targets for fungal inhibitors.

5 Still a further aspect is the finding that a yeast *skn7p* cognate also appears in other fungi, including *C. albicans* and *A. fumigatus*, and other filamentous fungi but does not appear in humans. Hence, the inventors have also identified the histidine kinase that phosphorylates *skn7p*
10 (*skn7p* kinase) and its cognates as another excellent target for potential antifungals.

 An additional feature, as more fully described below, is the discovery that the H-box and D-box domains of the newly identified osmosensing 2-component histidine kinases,
15 *osl*p (*N. crassa*) and *cos*lp (*C. albicans*), are highly conserved with respect to 2-component histidine kinases from yeast (*sln*lp) and bacteria (*BarA*, *RepA* and *ApdA*).

 Furthermore, the H-box domain of *cos*lp is identical to the H-box region of *osl*p.

20 The present invention includes an assay system for a broad range of 2-component histidine kinases, including fungal 2-component histidine kinases. The assay is based upon the two-step reaction that 2-component histidine kinase catalyze: namely, the autophosphorylation of a histidine
25 residue in the H-box and the subsequent transfer of the phosphate to an aspartate residue in the D-box or receiver domain. Hence in the assay, a 2-component histidine kinase catalyzes the reaction between a target substrate and a phosphoryl donor to form a phosphorylated target substrate.
30 This phosphorylated target substrate can be monitored by standard biochemical techniques. The preferred embodiment of the target substrate is a peptide. The choice of

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peptide is informed by the homology observed in the H-box and D-box receiver domains of 2-component histidine kinases as described above. In general, however, the sequence of the peptide is selected to mimic a portion of the H-box or D-box domain of the histidine kinase of interest. The preferred embodiment of the phosphoryl donor is ATP or a radiolabeled form thereof. The novel assay system has several advantages: 1) the assay does not require complex instrumentation; 2) it can be performed on a high throughput basis; and 3) diverse sample types can be assayed by the disclosed method, including for example, natural products, intact or extracts of microorganisms such as fungi and bacteria, and combinatorial or peptide libraries.

The invention further provides peptides capable of serving as substrates in assays for 2-component histidine kinases comprise a further aspect. These peptides are based upon the conserved sequences in the H-box and D-box domains found in newly identified *oslp* and *coslp*.

The histidine kinase assay system can also be used as a tool in the screening of a new class of fungal inhibitors. Screening can be accomplished using in vitro or whole-cell assay methods. Whereas most researchers working in the development of antifungals have attempted to identify inhibitors to enzymes involved in the synthesis of fungal cell-wall polymeric compounds, the present invention takes a new and different approach and describes an assay system for kinases involved in 2-component signal transduction pathways that play a critical role in fungal cell-wall assembly but which do not catalyze reactions involved in polymer synthesis. Consequently, the current invention permits the identification of a whole new class of fungal inhibitors. Although this invention describes the assay primarily with

regard to histidine kinases from fungi, the assay described herein has utility with histidine kinases from other sources such as bacteria.

5 BRIEF DESCRIPTION OF THE DRAWING

The Figure is a restriction map of the cosmid 12:4D containing approximately 35 kb of *Neurospora crassa* (*N. crassa*) DNA showing which restriction fragments were capable of complementing an *os-1* mutant.

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DETAILED DESCRIPTION OF INVENTION

Evidence demonstrating that *os-1* from *N. crassa* is a 2-component histidine kinase, that cognates exist in a broad range of fungi and bacteria and that humans lack a cognate, makes 2-component histidine kinases are excellent targets for fungal inhibitors. Evidence supporting the view that *os-1* is an osmosensing 2-component histidine kinase is two-fold. First, as more fully described in Example 1, the *os-1* gene has been isolated and sequenced by the inventors and the results show that there is significant homology between *os-1p* (SEQ ID NO: 1), the protein encoded by *os-1*, and *sln1p*, the yeast osmosensing 2-component histidine kinase, especially in the regions comprising the H-box, D-box, and ATP binding domains (the approximate sequence regions included in these domains are listed in Table I below). Secondly, osmotic mutants have an altered morphology when grown on media supplemented with 4% NaCl as compared to wild type, consistent with the mutants having a defective osmosensing 2-component histidine kinase. Details of these experiments are listed in Example 2.

Several lines of evidence demonstrate that *os-1* cognates exist in diverse fungi and bacteria but are absent

from humans. First, as just described, *os-1p* has significant homology to *slnlp* of *Saccharomyces cerevisiae* [Ota, I.M., and Varshavsky, A., Science 262:566-569 (1993)] (incorporated herein by reference). As discussed in Example 1, the *os-1* sequence is also homologous to the bacterial 2-component histidine kinases, BarA (bacterial adaptive response) of *Escherichia coli* [Morgan, B., et al., J. Cell Biol. 5:453-457 (1995); Nagasawa, et al., Mol. Microbiol. 6:799 (1992)], RepA (required for production of extra-cellular enzymes) of *Pseudomonas viridiflava* [Liao, C.-H., et al., Mol. Plant-Microbe Interact. 7:391-400 (1994)] and ApdA (antibiotic production) of *Pseudomonas fluorescense* [Corbell, N. and Loper, J.E., J. Bacteriol. 177:6230-6236 (1995)], (the foregoing four references incorporated herein by reference). Furthermore, the inventors have isolated and partially sequenced a gene which they call *cos1* (the deduced partial amino acid sequence is listed as SEQ ID NO: 2) from a second fungus, *C. albicans*, that is homologous to *os-1*. Finally, using a portion of the *N. crassa os-1* gene as a probe, Southern blot analyses were conducted on DNA isolated from a number of fungi, bacteria, plants and mammals according to procedures well known in the art. Hybridization bands were present in samples from *N. crassa* (as control), *C. albicans*, *A. fumigatus* and *E. coli*; hybridization bands were present in numerous other filamentous fungi as well. Importantly, hybridization with insect, mouse and human DNA was not detected, even at low stringency. In addition, when primers homologous to regions from the H-box and D-box domains from *N. crassa* were used (see Table I), polymerase chain reaction (PCR) products using human and other mammalian DNA as templates were not detected. Taken together, these results indicate that *os-1*

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cognates are not present in humans and that *os-1* function is unique to fungi and bacteria; hence, such 2-component histidine kinases would be good targets for broad-acting antifungal and antibacterial agents.

5 In addition to the overall homology between *os-1* and other fungal (*sln1* and *cos1*) and bacterial 2-component histidine kinases (BarA, RepA and AdpA), there is a particularly high degree of homology in 3 domains corresponding to the H-box, D-box and ATP binding site
10 domains of these proteins. The approximate sequence regions included in these domains are listed in Table I. The *os-1* H-box, ATP binding site and D-box are defined approximately by regions 698 to 843, 870 to 931 and 1093 to 1203, respectively. His⁷¹⁸ and Asp¹¹³⁶ are the presumed phosphoryl
15 acceptors. Hence, the invention involves, in part, the use of compounds designed to mimic certain portions of these regions as substrates in a 2-component histidine kinase assay system.

TABLE I
Regions of Protein Sequence Homolog

Protein	H-Box	ATP-Binding	
		Domain	D-Box
<i>osl</i> p	698-843	870-931	1093-1203
<i>Bar A</i>	282-427	451-512	674-782
<i>Rep A</i>	253-398	422-483	652-763
<i>Apd A</i>	274-419	443-504	673-784
<i>Sln</i> lp	556-703	859-920	1081-1207

For *os-1* sequence, see SEQ ID NO: 1; for other sequences, see references listed in text.

It has also been discovered from PCR amplification and hybridization experiments that are more fully described in Example 3 that yeast *skn7p* cognates exist in a variety of fungi but not in humans, suggesting that the hexokinase that phosphorylates *skn7p* (*skn7p* kinase) and its cognates would be another target for antifungals. In addition to finding that *skn7* cognates existed in diverse fungi, it was also discovered that residues 420 through 434 of *skn7p* (residue numbers are based upon the sequence as listed in Brown, J.L., et al. [*J. Bacteriol.* 175:6908-6915 (1993)] (incorporated herein by reference)) was homologous to certain regions of the D-box domains from *osl*p (residues 1129 through 1143), *sln*lp (yeast; residues 1137 through 1151) and *BarA* (bacteria; residues 711 through 725). This result indicates that compounds modeled after the *skn7p* D-box domain sequence may also have utility as substrates in 2-component hexokinase assays.

Finally, additional useful substrates for histidine kinases would include peptides which mimic the receiver domains of proteins which receive the phosphoryl group transferred from the phosphoaspartate residue of histidine

kinases. An example would include substrates which mimic portions of the D-box or receiver domain of *ssk1p* [Maeda, T., et al., *Nature* 369:242-45 (1994)] (incorporated herein by reference).

5 Having identified 2-component histidine kinases as novel targets for antifungals and antibacterial agents and further identifying amino acid sequences that could serve as the basis for selecting substrates, it was possible to design an assay for histidine kinases. The assay is based
10 upon the observation that 2-component histidine kinases first autophosphorylate a histidine residue located in the H-box using ATP as the phosphoryl donor and subsequently transfer the phosphoryl group in a second autophosphorylation reaction to an aspartate residue located
15 in the D-box. Finally, the phosphoryl group on the phosphoaspartate is transferred to the receiver domain of another protein, the "receiver protein" (for example, *ssk1p* in *S. cerevisiae*). The assay generally utilizes target substrates which mimic regions from the H-box and D-box
20 domains of the histidine kinase of interest or the D-box (receiver domain) of the receiver protein. Specifically, the invention describes an assay for *oslp*, its cognates and other histidine kinases which have homologous D-box or H-box domains, such as those listed above, using target substrates
25 which mimic regions from the H-box and D-box domains of newly identified *os-1* and *cos-1* (SEQ ID NOs: 3-10) and a portion of the D-box of receiver protein *ssk1p* (SEQ ID NO: 12). As described above, since the inventors have demonstrated that these regions are homologous with regions
30 in other diverse fungal and bacterial 2-component histidine kinases, the assay has broad utility. Target substrates are also designed to mimic a region from the D-box of *skn7p* (for

example, SEQ ID NO: 11). Since the inventors have demonstrated that *skn7p* cognates also exist in diverse fungi, assays utilizing target substrates modeled after this sequence would also have broad utility.

5 The method comprises preparing a reaction mixture containing histidine kinase or a sample potentially containing histidine kinase with a target substrate, metal ion and a phosphoryl donor. Phosphorylated target substrate generated during the reaction can be monitored by a variety
10 of techniques.

 The term "histidine kinase" is used broadly to refer to those enzymes which catalyze phosphoryl transfer from a phosphoryl donor such as ATP to a substrate which contains a histidine residue or aspartate residue. Such histidine
15 kinases include 2-component histidine kinases. Preferably, such 2-component histidine kinases include fungal and bacterial histidine kinases, especially *skn7p* kinase and the proteins encoded by *os-1* and *cos-1*.

 Histidine kinase may be substantially pure histidine
20 kinase or unpure histidine kinase. The term unpure means that the sample contains other compounds or proteins besides histidine kinase. Such sample may be taken from crude lysates of fungi and bacteria.

 As used in this invention, metal ion is meant to
25 generally include divalent metal ions, preferably Mg^{2+} .

 The term "target substrate" is meant to broadly refer to compounds capable of accepting a phosphate group, or analogue thereof, during the phosphoryl transfer reaction catalyzed by a histidine kinase such that phosphorylated
30 target substrate is produced. Such target substrate may include a peptide, especially a peptide containing a histidine and/or aspartic acid residue. If target substrate

comprises a peptide, such peptide is preferably includes an amino acid sequence selected from the group consisting of the histidine kinase's D-box domain, histidine kinase's H-box domain domain, and D-box domain of the receiver protein phosphorylated by the histidine kinase, a fragment thereof or an analogue thereof. If target peptide comprises a peptide, such peptide is more preferably comprised of less than 20 amino acid residues and includes at least one of an aspartate and histidine residue. In another embodiment, target substrate comprises at least 10 amino acids but less than 20 amino acids, and most preferably, wherein at least one such amino acid is a histidine or aspartic acid residue. In the most preferred embodiment, the target substrate is selected from the group consisting of SEQ ID NOs: 3-12, a fragment thereof, and an analogue thereof.

The term "fragment" is meant to mean a portion of the peptides listed in SEQ ID NOs: 3-12. The term analogue refers to peptides in which one or more of the amino acids of the peptides listed in SEQ ID NOs: 3-12 has been:

- 1)chemically derivatized and/or 2)substituted with one or more different amino acids. Both fragments and analogues remain functionally similar to those listed in that the ability of a histidine kinase to phosphorylate histidine or aspartate is substantially preserved.

The term "phosphoryl" donor generally includes compounds comprising triphosphate esters or an analogue of such triphosphate ester. In the preferred embodiment, such cosubstrate comprises ATP or an ATP analogue. ATP may be nonradioactive or radioactive, including for example, ^{32}P - γ -ATP or ^{33}P - γ -ATP. The term ATP analogue means molecules wherein one or more atoms of components of ATP (adenine, deoxyribose and triphosphate) has been substituted with a

different atom and/or wherein one or more of such components has been chemically derivatized, but in any case, the resulting molecule's ability to undergo phosphoryl transfer is substantially preserved.

5 Monitoring the formation of phosphorylated target substrate can be achieved using chromatography, radioassays and immunological methods that are well-known in the art. Of course, instead of monitoring the formation of phosphorylated target substrate, it would also be possible
10 to monitor the decrease in phosphoryl donor or target substrate to determine the concentration of histidine kinase as well.

Several embodiments of the invention utilize radioassays to determine the concentration of phosphorylated
15 target substrate formed and hence the concentration of histidine kinase present. For example, one embodiment of the invention utilizes the method essentially as described by Casnellie, J.E. [*Methods Enzymol.* 200:115-120] (incorporated herein by reference). As applied to this
20 invention, the monitoring procedure involves histidine kinase catalyzing a phosphoryl transfer reaction between phosphoryl donor and target substrate to generate phosphorylated target substrate. After terminating reactions, an aliquot of reaction mixture is spotted on a
25 filter, for example, a phosphocellulose P-81 filter, which is then washed with 0.5% phosphoric acid solution to remove unreacted radiolabeled ATP. Filters are dried, placed in scintillation vials with scintillation fluid and the amount of radioactivity determined by liquid scintillation
30 counting. Details are described in Example 5.

Another embodiment of the invention utilizes a radioactive technique using target substrate labeled with

biotin (biotinylated target substrate), preferably at the N-terminus if target substrate is a peptide, as described by Goueli, B.S. et al. *Anal. Biochem.* 225:10-17 (1995), incorporated herein by reference. Briefly, biotinylated
5 target substrate and ^{32}P - γ -ATP are used as substrates in the presence of histidine kinase. Reactions are terminated and filtered through a filter impregnated with streptavidin (for example, a streptavidin-impregnated nitrocellulose filter) which binds biotinylated phosphorylated target substrate and
10 unreacted biotinylated target substrate. Filters are washed to remove unincorporated radioactive ATP, dried and the amount of ^{32}P -labeled biotinylated phosphorylated target substrate determined using a PhosphorImager. Details of this approach are described in Examples 6 and 7.

15 Scintillation proximity detection may be used in another embodiment of the invention to monitor formation of phosphorylated target substrate according to the method described by Cook, Neil D., *Scintillation Proximity Assay: A Versatile High-Throughput Screening Technology*, DDT 1:287-
20 94, the foregoing reference and the references therein incorporated herein by reference. Briefly, as applied to this invention, scintillation proximity detection may be used to monitor reactions as follows. Reactions between biotinylated target substrate and ^{33}P - γ -ATP are run in the
25 presence of histidine kinase. Reactions are run in, or are transferred to, a collection device embedded with scintillant and streptavidin. Preferably, such collection device is a microtiter well. Streptavidin binds ^{33}P -labeled biotinylated target substrate. Collection device is washed
30 to remove unreacted ^{33}P - γ -ATP. Radioactive decay of the bound ^{33}P -labeled biotinylated target substrate causes the embedded scintillant to fluoresce; the fluorescence can be

monitored to determine concentration. As compared to the filter radioactive assays described above, this method has the advantage of permitting higher throughput. The method is more fully described in Example 8.

5 As a way of potentially avoiding the use of radioactive reagents, the invention also utilizes immunological methods involving antibodies to monitor formation of phosphorylated target substrate and thus the concentration of histidine kinase. Formation of complex and detecting complex can be
10 accomplished using a variety of methods which are well-known to those skilled in the art. Examples of such methods include: 1) direct precipitation of the antibody/
phosphorylated target substrate complex; 2) latex agglutination; 3) radioimmunoassay; or 4) enzyme-linked
15 immunosorbant assay (ELISA). In the most preferred embodiment, the invention utilizes monoclonal antibodies to detect phosphorylated target substrates by ELISA and is more fully described in Example 10. This embodiment has the advantage of not requiring radioactive materials.

20 The term "antibody" is used broadly to refer to antibodies which selectively bind phosphorylated target substrate to form a complex. Such antibody may recognize one or more epitopes of the phosphorylated target substrate. Alternatively, antibody may recognize and bind to
25 phosphohistidine or phosphoaspartate located within phosphorylated target substrate.

Antibody may be an entire antibody or an antigen binding fragment thereof; antibody may also belong to any immunoglobulin class. Furthermore, antibody may be a
30 natural antibody, i.e., derived from animal sources, or alternatively, may be a recombinant antibody, i.e., produced from recombinant DNA techniques. Antibody or antibody

fragment may be of polyclonal or monoclonal origin, but preferably is of monoclonal origin.

Antibodies used in this invention can be prepared using phosphorylated target substrate directly as antigen or, if of
5 insufficient size, as a hapten coupled to another protein utilizing standard methods that are well known in the art. For example, to produce polyclonal antibodies, phosphorylated target substrate may be injected into a suitable host to induce antibody production. Serum from
10 host is then collected and the desired polyclonal antibody purified by standard techniques. Monoclonal antibodies can be prepared according to the method of Kohler et al., *Eur. J. Immunol.* 6:511 (1976), herein incorporated by reference. Antibody fragments may be produced, for example, by
15 following the protocol of Parham, *J. Immunol.* 131:2895 (1983) or Lamoyi and Nisonoff, *J. Immunol. Meth.* 56: 235 (1983), the preceding two references incorporated herein by reference. Production of antibodies prepared by recombinant DNA methods can be achieved according to the methods
20 reviewed by Winter, et al., *Annu. Rev. Immunol.* 12:433-55 (1996), the foregoing reference and those included therein incorporated herein by reference. Antibodies thus generated may be labeled with a detectable label, or may be conjugated with an effector molecule or enzyme according to methods
25 which are well-known in the art. Detectable label may include, for example, radionucleotides, dyes or fluorescent compounds.

METHOD OF SCREENING INHIBITORS OF HISTIDINE KINASES

30 Another aspect of the invention is a method for screening inhibitors of histidine kinase activity. As mentioned earlier, this novel screening method has important

utility in aiding in the identification of a new class of broad-based antifungal and antibacterial agents. In one aspect of the screening method, inhibitors can be tested using *in vitro* samples; whereas in another aspect of the invention, inhibitors are tested by their ability to inhibit fungal growth, i.e., by a whole-cell assay method.

The *in vitro* method for screening inhibitors employs the histidine kinase assay system described above. A reaction mixture containing target substrate, metal ion and phosphoryl donor is prepared. Histidine kinase is added and a first rate of formation of phosphorylated target substrate determined according to the monitoring approaches described above. A second rate of formation of phosphorylated target substrate is determined in a reaction mixture containing target substrate, metal ion, phosphoryl donor and putative inhibitor. A putative inhibitor includes compounds that potentially would inhibit the catalytic activity of a histidine kinase. First rate of formation is compared to second rate of formation; a slower second rate indicates that the putative inhibitor is an actual inhibitor.

Reactions can be run in a single reaction mixture, in which case putative inhibitor would be added after establishing a first rate of formation in the absence of putative inhibitor. Alternatively, reactions can be run in separate reaction mixtures, i.e., in a first and second reaction mixture, the amount of phosphorylated target substrate determined according to the approaches described above and compared to control reactions lacking potential inhibitor. The method is described more fully in Example 10. Potential inhibitors that do in fact inhibit histidine kinase (i.e., those compounds which result in second rate of formation

being less than first rate of formation) can then be tested further for their ability to inhibit fungal growth.

The whole-cell method for screening inhibitors is based upon the fact that inhibitors of fungal 2-component

5 histidine kinases inhibit the autophosphorylation reaction which normally occurs when cells are grown on a medium of low osmolarity, i.e., a medium having an approximate concentration of less than 150 mM salts, (for example, NaCl), sorbitol, sorbose, glucose or mannitol.

10 Consequently, using *N. crassa* as an example, *osl*p would not phosphorylate its *ssk*lp cognate in the presence of an inhibitor of the 2-component histidine kinase. The unphosphorylated form of *ssk*lp is the active form which triggers the *Hog*lp osmolarity response. This is appropriate

15 if the cell is in high osmolar solutions. However, in low osmolar solutions, activation of the *Hog*lp enzymatic cascade is inappropriate and causes the cells to die. Hence, the whole-cell screening method comprises growing a culture of fungal cells (of known number) on a medium of low osmolarity

20 to which a putative inhibitor has not been added to determine a first growth rate. A second growth rate is determined for a culture of cells grown on a medium of low osmolarity to which a putative inhibitor has been added. First and second growth rates are compared. If the second

25 growth rate is slower than the first growth rate, this is evidence that the putative inhibitor is an actual inhibitor. Cell growth is monitored using an optical reader. In the preferred embodiment, the medium is placed in microtiter plates and the amount of cell growth measured using a

30 Molecular Device's microtiter optical plate reader to facilitate high throughput. The method is more fully described in Example 11.

KITS FOR ASSAYING HISTIDINE KINASES

The kit for assaying histidine kinases comprises a target substrate and a means for detecting phosphorylated target substrate. Target substrate may include any of the compounds described above, but most preferably is a peptide selected from the group consisting of SEQ ID NOs: 3-12. Means for detecting may include one of the filters described earlier which bind phosphorylated target substrate. Means for detecting may also include a collector device as described above which is impregnated with streptavidin and/or scintillant for use in scintillation proximity detection. Alternatively, means for detecting may include an antibody which selectively binds phosphorylated target substrate. As described above, antibody may be specific for one or more epitopes of phosphorylated target substrate and may be specific for phosphohistidine or phosphoaspartate. Antibody may also be conjugated to a detectable label, such as radionucleotides, dyes, enzymes, fluorescent compounds or biotin.

20

EXAMPLE 1

Cloning and Sequencing of the *os-1* Gene

I. Materials and Methods

25 A. Strains and Media:

The following *N. crassa* strains were obtained from the Fungal Genetics Stock Center (Kansas City, KS): wild type (74-OR8-1a), *os-1* (NM233t), *nik-1* (S1413), *os-1* (B135), *os-1* (P3282), *os-1* (UCLA 80), *os-2* (UCLA 80), *os-4* (NM201₀), *os-5* (NM216₀) and *cut* (LLM1). An *os-1* (NM233t) *nic-1* strain was constructed by crossing a temperature-sensitive osmotic mutant, *os-1* (NM233t)A, with *nic-1* (S1413)a (Selitrennikoff,

30

C., et al., *Exp. Mycol.* 5:155-161 (1981), incorporated herein by reference).

N. crassa stocks were grown at 25°C on agar-solidified Vogel's medium N [Vogel, H. J., *Microbiol. Gent. Bull.* 13:42 (1956)] plus 1.5% (w/v) sucrose (VMS medium). Strains requiring nicotinamide were grown in medium supplemented with nicotinamide at 10 µg/ml (VMSN medium). Benomyl (Dupont, Wilmington, DE) was added to cooled (45 °C) medium at a final concentration of 1 µg/ml. *Escherichia coli* strains XL1-Blue and TB-1 were routinely maintained on LB medium, as described by Sambrook, J.T., et al. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); incorporated herein by reference in its entirety.

15

B. Plasmids:

The plasmid, pM063, (a gift from Dr. M. Orbach) contains an allele of *tub-2*, that confers benomyl resistance, as a 3.1 kb *HindIII* DNA fragment in pUC118. pCE1 was constructed by self-ligation of a 13kb *HindIII* DNA fragment of cosmid 12:4D from the Vollmer-Yanofsky genomic library [Vollmer, S. J., and Yanofsky, C., *Proc. Natl. Acad. Sci. U.S.A.* 83:4869-4873 (1986), incorporated herein by reference]. A cosmid vector, pSV50, that confers benomyl resistance was used to construct the *N. crassa* genomic DNA library [Orbach, M. J., et al., *Mol. Cell. Biol.* 6:2452-2461 (1986); Vollmer, S.J. and Yanofsky, C., *Proc. Natl. Acad. Sci. U.S.A.* 83:4869-4873 (1986)] (both of the preceding references incorporated herein by reference). pCE1 contains an ampicillin resistance gene and the origin of replication from pSV50 (originally derived from pBR322) and 9.3 kb of genomic *N. crassa* DNA sequences. pCE5

contains a 6.5 kb *EcoRI*/*NotI* DNA fragment of pCE1 that was inserted into pBluescriptSK (Stratagene, La Jolla, CA) (the *EcoRI* site is in the polylinker of pCE1 adjacent to a *Sau3AI* site). pCE6 contains a 2.8 kb *NotI*/*HindIII* DNA fragment subcloned from pCE1. pMMS100 contains a 7.0 kb *EcoRI*/*SmaI* DNA fragment subcloned from pCE1 into pBluescriptSK. pMMS108 is a partial *XhoI* deletion of pMMS100 that contains a 4.6 kb *XhoI*/*SmaI* DNA insert in pBluescript SK.

10 II. Results

A. Subcloning of *os-1*⁺

The *os-1* gene was isolated from the Vollmer-Yanofsky *N. crassa* genomic library [Vollmer, S.J., and Yanofsky, C., *Proc. Natl. Acad. Sci. U.S.A.* 83:4869-4873 (1986)] (incorporated herein by reference) by a chromosome walk. The resulting cosmid, 12:4D, contains approximately 35 kb of DNA that functionally complemented an *os-1* mutant (Figure 1). To isolate and sequence the *os-1*⁺ gene, the smallest DNA fragment containing the *os-1*⁺ gene was subcloned. DNA of the cosmid 12:4D was digested with a variety of individual restriction enzymes, and the digests were transformed into competent *os-1*(NM233t) *nic-1* cells.

DNA-mediated transformations were done using the procedure described by Selitrennikoff and Sachs [*Fungal Genetics Newsl.* 38:92 (1991)] (incorporated herein by reference). Competent *N. crassa* spheroplasts were prepared using the procedure of Vollmer and Yanofsky *Proc. Natl. Acad. Sci. U.S.A.* 83:4869-4873 (1986); incorporated herein by reference. Competent *os-1*(NM233t) *nic-1* and *os-1*(B135) cells were transformed with the cosmid, 12:4D, or co-transformed with subclones of 12:4D and pSV50 or pMO63 at a molar ratio of approximately 5:1, respectively.

Transformant colonies were initially selected for benomyl resistance. After 2-3 days of growth, benomyl-resistant colonies were transferred to agar-solidified VMSN medium slants and grown for 2-3 days. Complementation of the *os-1* mutant salt-sensitive phenotype was scored on slants of agar-solidified VMSN medium supplemented with nicotinamide and 4% (w/v) NaCl. Transformants of the temperature-sensitive *os-1* mutant were grown at 37°C, whereas transformants of the non-temperature sensitive *os-1* mutant were grown at 26°C.

Transformants were selected for benomyl resistance and then tested for their ability to grow on minimal medium supplemented with 4% (w/v) NaCl. Introduction of a *Hind*III digest of 12:4 DNA into *os-1*(NM233t) *nic-1* cells resulted in several transformants that grew similar to wild-type on NaCl-supplemented medium at 37°C, suggesting that *Hind*III did not cut within the functional *os-1*⁺ gene. Subsequently, a *Hind*III DNA fragment of 12:4 D was subcloned as pCE1. pCE1 contains a 9.3 kb genomic DNA fragment that complemented the *os-1* mutant. A partial restriction map is shown in Figure 1.

To isolate an *os-1*⁺-containing fragment smaller than 9.3 kb, several DNA fragments were subcloned, and the subclones assayed for complementation of *os-1*(NM233t) *nic-1*. A *Not*I/*Hind*III deletion of pCE1, designated pCE5, was not able to complement *os-1* (Figure 1). Also, a cloned *Not*I/*Hind*III DNA fragment of pCE1 (pCE6) did not complement the *os-1* mutant, suggesting that the *Not*I site is within a functional part of the *os-1*⁺ gene. A *Sma*I/*Hind*III deletion of pCE1, designated pMMS100, complemented *os-1*(NM233t) *nic-1*, whereas a partial *Xho*I deletion of pMMS100 (pMMS108) did not complement, suggesting that the *os-1*⁺ gene is contained

within the 7.0 kb *Sau3A1/SmaI* genomic DNA fragment of pMMS100 (Figure 1). Furthermore, transformation of a non-temperature sensitive mutant, *os-1*(B135), with pMMS100 resulted in complementation of the salt-sensitive phenotype.

5 These complementation results indicated that a functional *os-1*⁺ gene was encoded on the genomic DNA fragment contained in pMMS100. To provide additional data to support this conclusion, the linear growth of pMMS100 transformants of *os-1* mutants and wild type were quantitated on agar-
10 solidified medium with and without 4% NaCl.

 Linear growth was measured in race tubes as described by Davis and DeSerres [*Methods Enzymol.* 27A:79-143 (1970)] (incorporated herein by reference) on agar-solidified VMSN medium supplemented with and without 4% NaCl (w/v). Race
15 tubes were constructed using 25 ml disposable pipettes (Fisher Scientific, Pittsburgh, PA) according to the procedure of White and Woodward [*Fungal Genetics Newsl.* 42:79 (1995)] (incorporated herein by reference). Growth
20 distances were measured relative to the origin of inoculation after 16, 24, 40, 48 and 64.5 hours of incubation at 37°C.

 Plots of the linear growth rate distance as a function of time showed that the growth rates of wild type, *os-1* mutants, and pMMS100-transformed *os-1* mutants were
25 essentially identical on medium without NaCl. On medium supplemented with 4% NaCl, pMMS100-transformed *os-1* mutants showed a restored osmotolerant phenotype, as evidenced by 18- and 26-fold differences between the growth
30 rates of the recipient strains, *os-1*(NM233t) *nic-1* and *os-1*(B135), and the pMMS100-transformed strains, MMS100t-16 and MMS100b-2, respectively. Taken together, these results

indicated that a functional *os-1* gene was located on the 7 kb genomic fragment of pMMS100.

C. DNA Sequencing and Analysis

5 Approximately 6.5 kb of the 7.0 kb DNA fragment of pMMS100 was sequenced on both strands from restriction sites *Bgl*II to *Sma*I (Figure 1). DNA sequencing was accomplished by primer walking and restriction fragment deletion. DNA sequencing and primer syntheses were done by DNA Services
10 (Cornell University, Ithaca, NY). Sequence analyses were performed using MacVector™ (Eastman Kodak Co., Rochester, NY), the basic local alignment search tools, PC/Gene (Inteligenetics, Campbell, CA), BLAST [Altschul, S.F., et al., *J. Mol. Biol.* 215:403-410 (1990)] and PROSITE [Bairoch,
15 A., *Nucleic Acids Res.* 21:3097-3103 (1993)] (the latter two references incorporated herein by reference).

Nucleotide sequence analysis indicated a predicted open reading frame (ORF) of approximately 4.1 kb that was interrupted by four introns. The *os-1* start of translation
20 was identified by sequence similarity to a *N. crassa*-specific start-of-translation consensus sequence, known as the Kozak sequence [Bruchez, J. J. P., et al., *Fungal Genetics Newsl.* 40:85-88 (1993)] (incorporated herein by reference). Similarly, introns were identified by searching
25 for *N. crassa*-specific, intron consensus sequences [Bruchez, J.J.P., et al., *Fungal Genetics Newsl.* 40:89-96 (1993)] (incorporated herein by reference). The introns ranged in size from 53 to 66 bp. Three sets of sequences similar to the *N. crassa*-specific start-of-transcription consensus
30 sequence were identified 714 to 881 bp upstream of the predicted start of translation.

Translation of the ORF predicted a 1298 amino acid protein (Oslp) (SEQ ID NO: 1) with a molecular mass of approximately 142 kDa and a calculated pI of 5.3. A BLAST comparison of Oslp to protein sequences in several databases indicated similarity with sensor histidine kinases of bacteria and yeast. Specifically, sequence similarity was noted between Oslp and BarA (bacterial adaptive response) of *Escherichia coli* [Morgan, B., et al., *J. Cell Biol.* 5:453-457 (1995); Nagasawa, et al., *Mol. Microbiol.* 6:799 (1992)], RepA (required for production of extra-cellular enzymes) of *Pseudomonas viridiflava* [Liao, C.-H., et al., *Mol. Plant-Microbe Interact.* 7:391-400 (1994)], ApdA (antibiotic production) of *Pseudomonas fluorescense* [Corbell, N. and Loper, J.E., *J. Bacteriol.* 177:6230-6236 (1995)], and Slnlp of *Saccharomyces cerevisiae* [Ota, I.M., and Varshavsky, A., *Science* 262:566-569 (1993)] (the foregoing five references incorporated herein by reference). The overall amino acid sequence identity of Oslp with BarA, RepA and ApdA is approximately 11%, whereas Slnlp shares about 7% identity.

As shown in Table I listed earlier, these proteins share three regions of significant homology. In the Oslp regions 698 to 843, 870 to 931, and 1093 to 1203, the overall amino acid sequence identity with the corresponding regions of BarA, RepA and ApdA is 40%, 45%, and 34%, respectively. Slnlp sequence identity with Oslp in these regions is 27%, 40% and 22%, respectively. These three domains are characteristic of histidine kinase and aspartate response regulator modules of signal-transduction proteins that couple environmental signals to adaptive responses [Morgan, B., et al., *J. Cell Biol.* 5:453-457 (1995); Perego, M. and Hoch, J., *Trends in Genetics* 12:97-101 (1996); Stock, J.B., et al., *Nature* 344:395-400 (1990)] (all three

of these references incorporated herein by reference). In particular, presumably *Oslp* region 698 to 843 comprises the H-box domain and region 1093 to 1203 the D-box domain; His⁷¹⁸ and Asp¹¹³⁶ are likely the two phosphoryl acceptors. *Oslp* also has a conserved ATP-binding motif, region 870 to 931, identical to the motif defined for bacterial and yeast response regulator modules [Ota, I.M., and Varshavsky, A., *Science* 262:566-569 (1993); Parkinson, J.S., and Kofoed, E.C., *Annu. Rev. Genet.* 26:71-112 (1992); Stock, J.B., and Lukat, G.S., *Annu. Rev. Biophys. Biophys. Chem.* 20:109-136 (1991)] (the foregoing three references incorporated herein by reference).

Additionally, the gene *cos-1* which the inventors recently isolated from *C. alibicans* has a very high degree of similarity with *os-1*. Approximately two-thirds of *cos-1* has been sequenced and a comparison of the deduced *oslp* sequence and the *coslp* sequence (SEQ ID NO: 2) shows that there is 65% identity and 83% similarity in the sequences.

20

EXAMPLE 2

Determination of Mutant Morphology
of Osmotic Mutants

I. Materials and Methods

25

A. Strains and Media:

Strains and Media were as described in Example 1.

II. Results

Osmotic mutants of *N. crassa*, when grown on slants of agar-solidified VMS, had a dense, cropped appearance compared to wild type [Perkins, D. D., et al., *Microbiol. Rev.* 46:426-570 (1982)] (incorporated herein by reference).

30

This morphology was particularly apparent with the cut mutant, whose aerial growth resembles a freshly cut surface. Osmotic mutants frequently had bright orange spots which appeared to be pockets of an as yet unidentified material, referred to as a "liquid exudate" by Grindle and Dolderson [Trans. Br. Mycol. Soc. 87:457-487 (1986)]. Taken together these observations suggest that the osmotic mutants have morphologically altered aerial hyphae.

In liquid VMSN medium, hyphae from each of the os mutants were similar to wild-type. However, in VMSN medium supplemented with 4% NaCl (w/v), the osmotic mutants had irregularly-shaped (rough and bumpy) hyphae compared to wild-type, and some hyphae resembled pseudoconidia. These observations indicate that the *osmotic* genes are important for maintaining normal cell morphology in high osmolarity medium.

EXAMPLE 3

PCR and Hybridization Results with *skn7* DNA

20

To determine whether cognates of the yeast gene *skn7* were present in other fungi, polymerase chain reaction (PCR) primers were designed that would amplify *skn7*-specific DNA. DNA from a number of different sources was used as template for PCR reactions under the following conditions: The forward primer was 5'-ccaccataaatagcaacgtc (SEQ ID NO: 13); the reverse primer was 5'-ggactctaaattctggatgc (SEQ ID NO: 14). PCR mixtures contained 2.5 μ M primer (each), 50 ng of genomic DNA, 10 mM dNTP and 1X polymerase buffer in a total volume of 50 μ L. Primers, buffers and nucleotides were mixed and heated at 94°C for 5 minutes in a Perkin-Elmer thermocycler and then cooled to 40°C for 5 minutes.

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TaqPolymerase (0.5 units) was added and mixtures cycled 30 times at 94°C (1 minute), 40°C (1 minute) and 72°C (2 minutes) followed by a 7 minute extension at 72°C.

Reactions were separated by agarose gel electrophoresis and stained with ethidium bromide. A duplicate gel was blotted and probed with radio labeled *skn7* DNA. Hybridization bands formed for yeast (control), *C. albicans* and *A. fumigatus*. In sharp contrast, bands were not found using human DNA as template. Only bands from yeast, *C. albicans* and *A. fumigatus* hybridized with the *skn7* DNA probe. These results indicate that cognates of yeast *skn7* are present in *C. albicans* and *A. fumigatus* and not in humans, thus suggesting that *skn7* histidine kinase would be a good target for antifungals.

15

EXAMPLE 4

Demonstration of the Assay's Functionality

Peptides having the sequence shown in SEQ ID Nos: 3 (portion of H-box region of *osl*p and *cos*lp), 6 (portion of D-box region of *os-1*) and 12 (portion of receiver domain of *ssk1*p) were synthesized commercially and then biotinylated at the N-terminus using standard biochemical techniques. Each peptide was dissolved in DMSO and stored at -20°C until used. Macroconidia of wild-type and a nontemperature-sensitive *osl* mutant of *N. crassa* were inoculated into minimal medium with and without 4% NaCl (w/v). Hyphae were harvested after 24 hours of growth, washed and treated with Novozym 234 (Sigma) for 30 minutes to form protoplasts. Protoplasts were harvested, washed, and lysed by resuspension in 50 mM HEPES, pH 7.5, containing 5 mM MgCl₂,

and 100 μ g/ml BSA. Lysates were centrifuged and low-speed supernatants used as enzyme sources.

Reaction mixtures contained 100 μ M ATP (~100,000 cpm 32 P- γ -ATP), 100 μ M of peptide (each peptide was used in a separate reaction) and 25 μ L cell extract in wells of a V-bottom 96-well microtiter plate. Reaction mixtures were incubated for 0 and 60 minutes at 25°C, and terminated by the addition of 5 μ L sample buffer containing 2% SDS (w/v), 1 mM DTT, 1% glycerol (w/v) and 0.1% bromphenol blue (v/v).

To determine whether peptides were phosphorylated, reaction products were separated by SDS-PAGE using tricine gels. Gels were fixed in 10% (v/v) glutaraldehyde, washed, wrapped in Saranwrap, exposed to a Phosphor screen overnight and the screens scanned the following day. Reaction mixtures without peptides, cell extracts or incubated for zero time did not have phosphorylated peptides. However, extracts of wild-type cells grown on a medium of low osmolarity were able to phosphorylate each peptide. Reaction mixtures lacking peptides did not show peptide phosphorylation. As expected, extracts of wild-type cells grown on a medium of high osmolarity were not able to phosphorylate peptide substrates as evidenced by the absence of radiolabeled peptide on the gels, presumably because *osl*, like *sln1p*, is only active at low osmolarity.

In contrast, extracts of an *osl* mutant grown on a medium of either low or high osmolarity phosphorylated peptide target substrates, thus resulting in a band of radiolabeled peptide being detected on gels. These results are consistent with the hypothesis that this *osl* mutant is constitutive, resulting in the inappropriate phosphorylation of a *ssk1p* homolog. This in turn results in the inability of mutant cells to derepress the *hog1* pathway, greatly

reducing growth in medium of high osmolarity. Collectively, these results demonstrate the functionality of this overall approach to assaying histidine kinases.

5

EXAMPLE 5

In vitro Assay Utilizing
Phosphocellulose Filters

Histidine kinase can be assayed in reaction mixtures
10 containing 100 μ M target substrate (most preferably a
peptide selected from the group consisting of SEQ ID NOS:3-
12), 100 μ M ATP (~100,000 cpm 32 P- γ -ATP) and 5 mM $MgCl_2$.
Reactions are initiated by addition of histidine kinase and
incubated at 25°C. Histidine kinase may be substantially
15 pure or from fungal extracts as described in Example 5.
Total reaction volume is approximately 25 μ l. At various
time points, reactions can be terminated by the addition of
5 μ l sample buffer containing 2% SDS (w/v), 1 mM DTT, 1%
glycerol (w/v) and 0.1% bromphenol blue (v/v). To monitor
20 formation of phosphorylated target substrate, an aliquot of
reaction mixture is spotted on a phosphocellulose filter,
preferably a phosphocellulose P-81 filter. Filters are
soaked in 0.5 % phosphoric acid for approximately 15
minutes, the acid decanted and the process repeated
25 approximately four times. Filters are dried and then placed
in scintillation vials containing scintillation fluid.
Radioactivity is determined using a liquid scintillation
counter. The amount of radioactivity is proportional to
concentration of phosphorylated target substrate and
30 indirectly the concentration of histidine kinase.

EXAMPLE 6

In vitro Assay Utilizing Streptavidin-Impregnated
Nitrocellulose Filters

5 Reaction mixture composition, reaction initiation and
reaction termination are essentially as described in Example
5. However, target substrate comprise a biotinylated target
substrate, most preferably a peptide selected from the group
consisting of SEQ ID NOs: 3-12 which have been biotinylated
10 at the N-terminus. Following termination of reaction, to
monitor formation of biotinylated phosphorylated target
substrate, each reaction mixture is then passed through a
filter impregnated with streptavidin (for example, those
manufactured by Prozyme) and previously blocked with bovine
15 serum albumin and 10 mM ATP to prevent nonspecific binding.
The biotinylated phosphorylated target substrate binds
strongly to the streptavidin-impregnated filter and is
retained, whereas unreacted radiolabeled ATP and extract
pass through the filter. Filters are washed three times
20 with 0.5 ml buffer (100 mM HEPES, pH 7.5, 100 µg/ml BSA and
5 mM MgCl₂) and then dried in a vacuum oven at 60°C for 30
minutes. Each filter is then wrapped in a single layer of
Saranwrap and exposed to a Phosphor screen. The amount of
radioactivity present can be quantified using a Molecular
25 Dynamics PhosphorImager. Although both biotinylated
phosphorylated target substrate and biotinylated
unphosphorylated target substrate bind to the streptavidin-
impregnated filters, only the phosphorylated form is
radiolabeled and thus detected.

EXAMPLE 7

In vitro Assay Utilizing Streptavidin-Impregnated
Nitrocellulose Filters in Microtiter Apparatus

5 The method is essentially described in Example 6.
However, after reactions have been terminated, to monitor
formation of biotinylated phosphorylated target substrate,
an aliquot of reaction mixture is transferred to separate
wells of a Milliblot D apparatus containing nitrocellulose
10 filters impregnated with streptavidin and previously blocked
with bovine serum albumin and 10 mM ATP to prevent
nonspecific binding. Wells are washed three times with 0.5
ml buffer (100 mM HEPES, pH 7.5, 100 µg/ml BSA and 5 mM
MgCl₂) and filters dried in a vacuum oven at 60°C for 30
15 minutes. Each filter is then wrapped in a single layer of
Saranwrap and exposed to a Phosphor screen. The amount of
radioactivity present in each well can be quantified using a
Molecular Dynamics PhosphorImager. This method has the
advantage of allowing very high sample throughput.

20

EXAMPLE 8

In vitro Assay Utilizing Scintillation
Proximity Technology

25 Reaction mixture composition, reaction initiation and
reaction termination are essentially as described in Example
5. However, ³³P-γ-ATP is substituted for ³²P-γ-ATP and
biotinylated target substrates similar to those described in
Example 6 are utilized. Treatment of the samples is
30 according to the methods reviewed by Cook, Neil D.
[Scintillation Proximity Assay: A Versatile High-Throughput
Screening Technology, DDT 1:287-294 (the preceeding

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reference and those listed therein incorporated by reference). An aliquot of reaction mixture is transferred to microtiter wells that are embedded at the bottom with scintillant and streptavidin. Alternatively, reactions can
5 be run directly in the well. Streptavidin binds the biotinylated target substrate. Wells are washed 3 times with 250 μ l of buffer similar to that described in Example 7 to remove unreacted ^{33}P - γ -ATP. The radiodecay of the radiolabel causes the scintillant to fluoresce; the
10 fluorescence can be monitored by a fluorimeter to determine concentration of phosphorylated target substrate. This method has the combined advantage of avoiding the use of filters and high sample throughput.

15

EXAMPLE 9

In vitro Assay Utilizing ELISA

Reaction mixture composition, reaction initiation and reaction termination are essentially as described in Example
20 5. The amount of phosphorylated target substrate is monitored by Enzyme-Linked Immunosorbant Assay (ELISA). An aliquot of reaction mixture is applied to immobilized support (preferably polystyrene) which has attached to it antibodies which selectively bind phosphorylated target
25 substrate, including for example, antibodies specific for phosphohistidine or phosphoaspartate. Antibodies bind phosphorylated target substrate to form a complex. Detection of complex is accomplished by reacting complex with a second antibody which selectively binds another
30 portion of phosphorylated target substrate and which is conjugated to an easily assayed enzyme. For example, the conjugated enzyme could be horse radish peroxidase

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[Ishikawa, et al., *J. Immunoassay* 4:209-327 (1983); Imagawa, M., et al., *J. Appl. Biochem.* 4:41-57 (1982) (both references and those listed therein incorporated by reference)] and its substrate could be, for example,
5 3,3',5,5'-tetramethylbenzidine [Josephy, P., et al., *J. Biol. Chem.* 257:3669-3675 (1982)]. Unbound second antibody is removed by washing. The enzyme conjugated to second antibody is assayed, thereby providing a measure of the concentration of phosphorylated target substrate and
10 indirectly the concentration of histidine kinase.

EXAMPLE 10

In vitro Method for Screening
Antifungal Inhibitors

15

Putative inhibitors are dissolved in 100% DMSO and added to reaction mixtures similar to those described in Example 5. Reactions are initiated by adding histidine kinase, which may be present in essentially pure form or as
20 an extract from cells. Reactions are incubated for various times and the amount of phosphorylated target substrate formed determined according to one of the monitoring methods described in Examples 5 through 8 above and compared to controls (DMSO alone).

25

EXAMPLE 11

Whole-cell Assay Method for Screening
Antifungal Inhibitors

30

Compounds which prove capable of inhibiting histidine kinase in the *in vitro* assay can be tested further for their ability to inhibit the growth of the human pathogenic fungi

C. albicans and *A. fumigatus*. Samples can be tested using microtiter broth assays.

1. Procedure for *C. albicans*: The microtiter broth assay procedure is a modification of the National Committee
5 for Clinical Laboratory Standards protocol M-27T. Liquid medium (200 μ L RPMI 1640 with glutamine and phenol red, without bicarbonate, buffered with MOPS to pH 7.0) in 96-well microtiter plates are inoculated with 2000 cells per well from an overnight culture. Putative inhibitors to be
10 tested are added to each well and plates incubated at 37°C for 48 hours. Amphotericin B (0.015 to 16 μ g/ml), fluconazole (0.06 to 64 μ g/ml), various amounts of each medium and DMSO can be used as controls. The growth rate can be determined using a Molecular Devices microtiter
15 optical plate reader at 690 nm.

2. Procedure for *A. fumigatus*: Liquid medium as described for *C. albicans* are inoculated with 2000 conidia per well from -80°C glycerol stocks. Compounds to be tested are added to each well and plates incubated at 37°C for 48
20 hours. Amphotericin B (0.015 to 16 μ g/ml), miconazole (0.06 to 64 μ g/ml), and DMSO can be used as controls.

3. Testing of samples for mammalian cell toxicity: Each sample that is found to inhibit one of the *in vitro* kinase assays and to inhibit fungal growth can be tested for
25 its effect on the growth of each of two mammalian cell lines. Cell lines can be obtained from the ATCC, including: COS-7 (ATCC #CRL 1651, Kidney, SV40 transformed, African green monkey) and SK-HEP-1 (ATCC #HTB 52, Adeno-carcinoma, liver, ascites, human). Cell lines will be grown at 37°C
30 with 5% CO₂ in a tissue culture incubator: COS-7 can be grown in Dulbecco's Modified Eagle Medium (Gibco BRL) and SK-HEP-1 can be grown in Minimum Essential Medium (Gibco

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BRL) plus sodium pyruvate. Both media can be supplemented with 10% Calf Serum (Gibco BRL). Cell lines can be cultivated in T25 tissue culture flasks and these can be subcultured 1:4 at weekly intervals.

5 Samples can be added to wells of 96-well microtiter plates containing 5×10^3 cells per well in 150 μ L medium. Plates can be incubated for 48 hrs at 37°C and the amount of growth in each well compared to controls (growth will be quantitated using a microtiter plate reader).

10 The foregoing examples are only meant to be illustrative of certain aspects of the current invention but are not meant to limit the invention to the specific embodiments listed. As those skilled in the art would readily recognize, it would be possible to make
15 modifications to certain aspects of the invention which would fall within the broad scope of this invention.

EQUIVALENTS

While this invention has been particularly shown and
20 described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art
25 will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

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- (ii) TITLE OF INVENTION: Histidine Kinase Assay
- (iii) NUMBER OF SEQUENCES: 14
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- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/832,617
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1298 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Neurospora crassa*
- (B) STRAIN: os-1(NM233t)nic-1

(vii) IMMEDIATE SOURCE:

- (B) CLONE: os-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Thr Asp Gly Pro Thr Leu Ala Ala Ile Ala Ala Leu Val Lys Ser
1 5 10 15

Leu Ala Val Asp Pro Ala Thr Thr Gln Thr Ser Gly Leu Arg Pro Ser
20 25 30

Thr His Val Arg Leu Pro Gly Pro Tyr Thr Arg Glu Lys Gly Asp Leu
35 40 45

Glu Arg Glu Leu Ser Ala Leu Val Val Arg Ile Glu Gln Leu Glu Thr
50 55 60

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Ala Ala Ile Ala Ala Ser Pro Pro Ala Met Pro Asp Thr Pro Asn Ala
65 70 75 80

Pro Thr Asp Ala Leu Phe Ser Asn Gly Thr Leu Ser Pro Ser Ser Glu
85 90 95

Thr Pro Asp Ala Arg Tyr Pro Ala Pro Leu Pro Arg Asn Gly Phe Ile
100 105 110

Asp Glu Ala Leu Glu Gly Leu Arg Glu His Val Asp Asp Gln Ser Lys
115 120 125

Leu Leu Asp Ser Gln Arg Gln Glu Leu Ala Gly Val Asn Ala Gln Leu
130 135 140

Ile Glu Gln Lys Gln Leu Gln Glu Lys Ala Leu Ala Ile Ile Glu Gln
145 150 155 160

Glu Arg Val Ala Thr Leu Glu Arg Glu Leu Trp Lys His Gln Lys Ala
165 170 175

Asn Glu Ala Phe Gln Lys Ala Leu Arg Glu Ile Gly Glu Ile Val Thr
180 185 190

Ala Val Ala Arg Gly Asp Leu Ser Lys Lys Val Arg Met Asn Ser Val
195 200 205

Glu Met Asp Pro Glu Ile Thr Thr Phe Lys Arg Thr Ile Asn Thr Met
210 215 220

Met Asp Gln Leu Gln Val Phe Ser Ser Glu Val Ser Arg Val Ala Arg
225 230 235 240

Glu Val Gly Thr Glu Gly Ile Leu Gly Gly Gln Ala Gln Ile Glu Gly
245 250 255

Val Asp Gly Thr Trp Lys Glu Leu Thr Asp Asn Val Asn Val Met Ala
260 265 270

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Gln Asn Leu Thr Asp Gln Val Arg Glu Ile Ala Ser Val Thr Thr Ala
275 280 285

Val Ala His Gly Asp Leu Thr Lys Lys Ile Glu Arg Pro Ala Lys Gly
290 295 300

Glu Ile Leu Gln Leu Gln Gln Thr Ile Asn Thr Met Val Asp Gln Leu
305 310 315 320

Arg Thr Phe Ala Ser Glu Val Thr Arg Val Ala Arg Asp Val Gly Thr
325 330 335

Glu Gly Ile Leu Gly Gly Gln Ala Asp Val Glu Gly Val Gln Gly Met
340 345 350

Trp Asn Glu Leu Thr Val Asn Val Asn Ala Met Ala Asn Asn Leu Thr
355 360 365

Thr Gln Val Arg Asp Ile Ile Lys Val Thr Thr Ala Val Ala Lys Gly
370 375 380

Asp Leu Thr Gln Lys Val Gln Ala Glu Cys Arg Gly Glu Ile Phe Glu
385 390 395 400

Leu Lys Lys Thr Ile Asn Ser Met Val Asp Gln Leu Gln Gln Phe Ala
405 410 415

Arg Glu Val Thr Lys Ile Ala Arg Glu Val Gly Thr Glu Gly Arg Leu
420 425 430

Gly Gly Gln Ala Thr Val His Asp Val Gln Gly Thr Trp Arg Asp Leu
435 440 445

Thr Glu Asn Val Asn Gly Met Ala Met Asn Leu Thr Thr Gln Val Arg
450 455 460

Glu Ile Ala Lys Val Thr Thr Ala Val Ala Lys Gly Asp Leu Thr Lys
465 470 475 480

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Lys Ile Gly Val Glu Val Gln Gly Glu Ile Leu Asp Leu Lys Asn Thr
485 490 495

Ile Asn Thr Met Val Asp Arg Leu Gly Thr Phe Ala Phe Glu Val Ser
500 505 510

Lys Val Ala Arg Glu Val Gly Thr Asp Gly Thr Leu Gly Gly Gln Ala
515 520 525

Gln Val Asp Asn Val Glu Gly Lys Trp Lys Asp Leu Thr Glu Asn Val
530 535 540

Asn Thr Met Ala Ser Asn Leu Thr Ser Gln Val Arg Gly Ile Ser Thr
545 550 555 560

Val Thr Gln Ala Ile Ala Asn Gly Asp Met Ser Arg Lys Ile Glu Val
565 570 575

Glu Ala Lys Gly Glu Ile Leu Ile Leu Lys Glu Thr Ile Asn Asn Met
580 585 590

Val Asp Arg Leu Ser Ile Phe Cys Asn Glu Val Gln Arg Val Ala Lys
595 600 605

Asp Val Gly Val Asp Gly Ile Met Gly Gly Gln Ala Asp Val Ala Gly
610 615 620

Leu Lys Gly Arg Trp Lys Glu Ile Thr Thr Asp Val Asn Thr Met Ala
625 630 635 640

Asn Asn Leu Thr Ala Gln Val Arg Ala Phe Gly Asp Ile Thr Asn Ala
645 650 655

Ala Thr Asp Gly Asp Phe Thr Lys Leu Val Glu Val Glu Ala Ser Gly
660 665 670

Glu Met Asp Glu Leu Lys Lys Lys Ile Asn Gln Met Val Tyr Asn Leu
675 680 685

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Arg Asp Ser Ile Gln Arg Asn Thr Gln Ala Arg Glu Ala Ala Glu Leu
690 695 700

Ala Asn Lys Thr Lys Ser Glu Phe Leu Ala Asn Met Ser His Glu Ile
705 710 715 720

Arg Thr Pro Met Asn Gly Ile Ile Gly Met Thr Gln Leu Thr Leu Asp
725 730 735

Thr Asp Leu Thr Gln Tyr Gln Arg Glu Met Leu Asn Ile Val Asn Ser
740 745 750

Leu Ala Asn Ser Leu Leu Thr Ile Ile Asp Asp Ile Leu Asp Leu Ser
755 760 765

Lys Ile Glu Ala Arg Arg Met Val Ile Glu Glu Ile Pro Tyr Thr Leu
770 775 780

Arg Gly Thr Val Phe Asn Ala Leu Lys Thr Leu Ala Val Lys Ala Asn
785 790 795 800

Glu Lys Phe Leu Asp Leu Thr Tyr Arg Val Asp His Ser Val Pro Asp
805 810 815

His Val Val Gly Asp Ser Phe Arg Leu Arg Gln Ile Ile Leu Asn Leu
820 825 830

Val Gly Asn Ala Ile Lys Phe Thr Glu His Gly Glu Val Ser Leu Thr
835 840 845

Ile Gln Lys Ala Ser Ser Val Gln Cys Ser Thr Glu Glu Tyr Ala Ile
850 855 860

Glu Phe Val Val Ser Asp Thr Gly Ile Gly Ile Pro Ala Asp Lys Leu
865 870 875 880

Asp Leu Ile Phe Asp Thr Phe Gln Gln Ala Asp Gly Ser Met Thr Arg
885 890 895

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Lys Phe Gly Gly Thr Gly Leu Gly Leu Ser Ile Ser Lys Arg Leu Val
900 905 910

Asn Leu Met Gly Gly Asp Val Trp Val Lys Ser Glu Tyr Gly Lys Gly
915 920 925

Ser Lys Phe Phe Phe Thr Cys Val Val Arg Leu Ala Asn Asp Asp Ile
930 935 940

Ser Leu Ile Ala Lys Gln Leu Asn Pro Tyr Lys Ser His Gln Val Leu
945 950 955 960

Phe Ile Asp Lys Gly Arg Thr Gly His Gly Pro Glu Ile Ala Lys Met
965 970 975

Leu His Gly Leu Gly Leu Val Pro Ile Val Val Asp Ser Glu Arg Asn
980 985 990

Pro Ala Leu Glu Lys Ala Arg Ala Ala Gly Gln Ala Pro Tyr Asp Val
995 1000 1005

Ile Ile Val Asp Ser Ile Glu Asp Ala Arg Arg Leu Arg Ser Val Asp
1010 1015 1020

Asp Phe Lys Tyr Leu Pro Ile Val Leu Leu Ala Pro Val Val His Val
1025 1030 1035 1040

Ser Leu Lys Ser Cys Leu Asp Leu Gly Ile Thr Ser Tyr Met Thr Thr
1045 1050 1055

Pro Cys Gln Leu Ile Asp Leu Gly Asn Gly Met Val Pro Ala Leu Glu
1060 1065 1070

Asn Arg Ala Thr Pro Ser Leu Ala Asp Asn Thr Lys Ser Phe Glu Ile
1075 1080 1085

Leu Leu Ala Glu Asp Asn Thr Val Asn Gln Arg Leu Ala Val Lys Ile
1090 1095 1100

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Leu Glu Lys Tyr His His Val Val Thr Val Val Gly Asn Gly Glu Glu
1105 1110 1115 1120

Ala Val Glu Ala Val Lys Arg Lys Lys Phe Asp Val Ile Leu Met Asp
1125 1130 1135

Val Gln Met Pro Ile Met Gly Gly Phe Glu Ala Thr Ala Lys Ile Arg
1140 1145 1150

Glu Tyr Glu Arg Ser Leu Gly Ser Gln Arg Thr Pro Ile Ile Ala Leu
1155 1160 1165

Thr Ala His Ala Met Met Gly Asp Arg Glu Lys Cys Ile Gln Ala Gln
1170 1175 1180

Met Asp Glu Tyr Leu Ser Lys Pro Leu Gln Gln Asn His Leu Ile Gln
1185 1190 1195 1200

Thr Ile Leu Lys Cys Ala Thr Leu Gly Gly Gln Leu Leu Glu Lys Asn
1205 1210 1215

Arg Glu Arg Glu Leu Thr Arg Ala Ala Asp Ala Val Thr Gly Gly Arg
1220 1225 1230

Arg Asp Asn Gly Met Tyr Ser Ala Ser Gln Ala Ala Gln His Ala Ala
1235 1240 1245

Leu Arg Pro Pro Leu Ala Thr Arg Gly Leu Thr Ala Ala Asp Ser Leu
1250 1255 1260

Val Ser Gly Leu Glu Ser Pro Ser Ile Val Thr Ala Asp Lys Glu Asp
1265 1270 1275 1280

Pro Leu Ser Arg Ala Arg Ala Ser Leu Ser Glu Pro Asn Ile His Lys
1285 1290 1295

Ala Ser

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 718 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Candida albicans

(vii) IMMEDIATE SOURCE:

(B) CLONE: cos-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asn Pro Thr Lys Lys Pro Arg Leu Ser Pro Met Gln Pro Ser Val
1 5 10 15

Phe Ile Ile Leu Asn Asp Pro Glu Leu Tyr Ser Gln His Cys His Ser
20 25 30

Leu Arg Glu Thr Leu Leu Asp His Phe Asn His Gln Ala Thr Leu Ile
35 40 45

Asp Thr Tyr Glu His Glu Leu Glu Lys Ser Lys Asn Ala Asn Lys Ala
50 55 60

Phe Gln Gln Ala Leu Ser Glu Ile Gly Thr Val Val Ile Ser Val Ala
65 70 75 80

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Met Gly Asp Leu Ser Lys Lys Val Glu Ile His Thr Val Glu Asn Asp
 85 90 95

Pro Glu Ile Leu Lys Val Lys Ile Thr Ile Asn Thr Met Met Asp Gln
 100 105 110

Leu Gln Thr Phe Ala Asn Glu Val Thr Lys Val Ala Thr Glu Val Ala
 115 120 125

Asn Gly Glu Leu Gly Gly Gln Ala Lys Asn Asp Gly Ser Val Gly Ile
 130 135 140

Asn Arg Ser Leu Thr Asp Asn Val Asn Ile Met Ala Leu Asn Leu Thr
 145 150 155 160

Asn Gln Val Arg Glu Ile Ala Asp Val Thr Arg Ala Val Ala Gln Gly
 165 170 175

Asp Leu Ser Arg Lys Ile Asn Val His Ala Gln Gly Glu Ile Leu Gln
 180 185 190

Leu Gln Arg Thr Ile Asn Thr Met Val Asp Gln Leu Arg Thr Phe Ala
 195 200 205

Phe Glu Val Ser Lys Val Ala Arg Asp Val Gly Val Leu Gly Ile Leu
 210 215 220

Gly Gly Gln Ala Leu Ile Glu Asn Val Glu Gly Ile Trp Glu Glu Leu
 225 230 235 240

Thr Asp Asn Val Asn Ala Met Ala Leu Asn Leu Thr Thr Gln Val Arg
 245 250 255

Asn Ile Ala Asn Val Thr Thr Ala Val Ala Lys Gly Asp Leu Ser Lys
 260 265 270

Lys Val Thr Ala Asp Cys Lys Gly Glu Ile Leu Asp Leu Lys Leu Thr
 275 280 285

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Ile Asn Gln Met Val Asp Arg Leu Gln Asn Phe Ala Leu Ala Val Thr
290 295 300

Thr Leu Ser Arg Glu Val Gly Thr Leu Gly Ile Leu Gly Gly Gln Ala
305 310 315 320

Asn Val Gln Asp Val Glu Gly Ala Trp Lys Gln Val Thr Glu Asn Val
325 330 335

Asn Leu Met Ala Thr Asn Leu Thr Asn Gln Val Arg Ser Ile Ala Thr
340 345 350

Val Thr Thr Ala Val Ala His Gly Asp Leu Ser Gln Lys Ile Asp Val
355 360 365

His Ala Gln Gly Glu Ile Leu Gln Leu Lys Asn Thr Ile Asn Lys Met
370 375 380

Val Asp Ser Leu Gln Leu Phe Ala Ser Glu Val Ser Lys Val Ala Gln
385 390 395 400

Asp Val Gly Ile Asn Gly Lys Leu Gly Ile Gln Ala Gln Val Ser Asp
405 410 415

Val Asp Gly Leu Trp Lys Glu Ile Thr Ser Asn Val Asn Thr Met Ala
420 425 430

Ser Asn Leu Thr Ser Gln Val Arg Ala Phe Ala Gln Ile Thr Ala Ala
435 440 445

Ala Thr Asp Gly Asp Phe Thr Arg Phe Ile Thr Val Glu Ala Leu Gly
450 455 460

Glu Met Asp Ala Leu Lys Thr Lys Ile Asn Gln Met Val Phe Asn Leu
465 470 475 480

Arg Glu Ser Leu Gln Arg Asn Thr Ala Ala Arg Glu Ala Ala Glu Leu
485 490 495

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Ala Asn Ser Ala Lys Ser Glu Phe Leu Ala Asn Met Ser His Glu Ile
500 505 510

Arg Thr Pro Leu Asn Gly Ile Ile Gly Met Thr Gln Leu Ser Leu Asp
515 520 525

Thr Glu Leu Thr Gln Tyr Gln Arg Glu Met Leu Ser Ile Val His Asn
530 535 540

Leu Ala Asn Ser Leu Leu Thr Ile Ile Asp Asp Ile Leu Asp Ile Ser
545 550 555 560

Lys Ile Glu Ala Asn Arg Met Thr Val Glu Gln Ile Asp Phe Ser Leu
565 570 575

Arg Gly Thr Val Phe Gly Ala Leu Lys Thr Leu Ala Val Lys Ala Ile
580 585 590

Glu Lys Asn Leu Asp Leu Thr Tyr Gln Cys Asp Ser Ser Phe Pro Asp
595 600 605

Asn Leu Ile Gly Asp Ser Phe Arg Leu Arg Gln Val Ile Leu Asn Leu
610 615 620

Ala Gly Asn Ala Ile Lys Phe Thr Lys Glu Gly Lys Val Ser Val Ser
625 630 635 640

Val Lys Lys Ser Asp Lys Met Val Leu Asp Ser Lys Leu Leu Leu Glu
645 650 655

Val Cys Val Ser Asp Thr Gly Ile Gly Ile Glu Lys Asp Lys Leu Gly
660 665 670

Leu Ile Phe Asp Thr Phe Cys Gln Ala Asp Gly Ser Thr Thr Arg Lys
675 680 685

Phe Gly Gly Thr Gly Leu Gly Leu Ser Ile Ser Lys Gln Leu Ile His
690 695 700

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Leu Met Gly Gly Glu Ile Trp Val Thr Ser Glu Tyr Gly Ser
705 710 715

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Neurospora crassa*

(vii) IMMEDIATE SOURCE:

(B) CLONE: os-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Asn Met Ser His Glu Ile Arg Thr
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(vii) IMMEDIATE SOURCE:

(B) CLONE: sln1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Asn Ile Ser His Glu Leu Arg Thr

1

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Escherichia coli*

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(vii) IMMEDIATE SOURCE:

(B) CLONE: BarA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Asn Met Ser His Glu Leu Arg Thr
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Neurospora crassa*

(vii) IMMEDIATE SOURCE:

(B) CLONE: os-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Ile Leu Met Asp Val Gln Met
1 5

(2) INFORMATION FOR SEQ ID NO:7:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Escherichia coli*

(vii) IMMEDIATE SOURCE:

(B) CLONE: Bar A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Ile Leu Met Asp Ile Gln Met
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas viridiflava*

(vii) IMMEDIATE SOURCE:

(B) CLONE: Rep A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Val Leu Met Asp Val Gln Met
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(vii) IMMEDIATE SOURCE:

(B) CLONE: sln1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Ile Phe Met Asp Val Gln Met
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas fluorescens*

(vii) IMMEDIATE SOURCE:

(B) CLONE: ApdA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Val Met Met Asp Val Gln Met

1

5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(vii) IMMEDIATE SOURCE:

(B) CLONE: skn7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Val Leu Met Asp Ile Val Met

1

5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(Å) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

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(A) ORGANISM: *Saccharomyces cerevisiae*

(vii) IMMEDIATE SOURCE:

(B) CLONE: ssklp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly	Leu	His	Leu	Ile	Phe	Met	Asp	Leu	Gln
1			5						10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Forward PCR primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCACCATAAA TAGCAACGTC

20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(A) DESCRIPTION: /desc = "Reverse PCR primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGACTCTAAA TTCTGGATGC

20

What is claimed is:

1. A method for assaying histidine kinase, comprising the steps of:

- 5 (a) preparing a reaction mixture in which a target substrate is phosphorylated in the presence of a phosphoryl donor and said histidine kinase to yield phosphorylated target substrate; and
- 10 (b) monitoring formation of said phosphorylated target substrate in said reaction mixture to determine concentration of said histidine kinase.

2. A method for screening for inhibitors of histidine kinases comprising the steps of:

- 15 (a) phosphorylating a target substrate in the presence of a phosphoryl donor and said histidine kinase in a reaction mixture to yield a phosphorylated target substrate and determining a first rate of formation of said phosphorylated target substrate;
- 20 (b) phosphorylating said target substrate in the presence of said phosphoryl donor, said histidine kinase and a putative inhibitor of said histidine kinase in a reaction mixture to yield a phosphorylated target substrate and determining a
- 25 second rate of formation of said phosphorylated target substrate; and
- 30 (c) comparing said first rate of formation with said second rate of formation to determine whether said putative inhibitor is an actual inhibitor of said histidine kinase.

3. The method according to Claim 1 or Claim 2 wherein said target substrate is a biotinylated target substrate and wherein said phosphorylated target substrate is a biotinylated phosphorylated target substrate.
- 5 4. The method according to any one of Claims 1 to 3 wherein said histidine kinase is a 2-component histidine kinase.
- 10 5. The method according to Claim 4 wherein said 2-component histidine kinase is a 2-component histidine kinase from a fungus.
- 15 6. The method according to Claim 5 wherein said fungus is selected from the group consisting of *Neurospora crassa*, *Candida albicans* and *Aspergillus fumigatus*.
7. The method according to any one of Claims 1 to 6 wherein said target substrate includes a peptide.
- 20 8. The method according to any one of Claims 1 to 7 wherein said target substrate comprises a peptide having at least one of an aspartic acid residue and a histidine residue.
- 25 9. The method according to any one of Claims 1 to 8 wherein said peptide includes fewer than 20 amino acids.
10. The method according to any one of Claims 1 to 9 wherein said peptide comprises more than 10 amino acids.
- 30 11. The method according to any one of Claims 1 to 10 wherein said peptide is selected from the group of amino

acid sequences consisting of SEQ ID NOs: 3-12, a fragment thereof, and an analogue thereof.

12. The method according to any one of Claims 1 to 11
5 wherein said phosphoryl donor is 32 P- γ -ATP and wherein said step of determining comprises:
- (a) transferring an aliquot of said reaction mixture to a filter which binds said phosphorylated target substrate;
 - 10 (b) washing said filter to remove said 32 P- γ -ATP; and
 - (c) determining amount of said phosphorylated target substrate bound to said filter.
13. The method according to Claim 12 wherein said filter is
15 selected from the group consisting of phosphocellulose, nitrocellulose, nylon and nytran.
14. The method according to any one of Claims 1 to 11
wherein said phosphoryl donor includes 32 P- γ -ATP and
20 wherein said step of monitoring or determining comprises:
- (a) transferring an aliquot of said reaction mixture to a streptavidin-impregnated filter which binds said biotinylated phosphorylated target substrate;
 - 25 (b) washing said streptavidin-impregnated filter to remove said 32 P- γ -ATP; and
 - (c) determining amount of said biotinylated phosphorylated target substrate bound to said streptavidin-impregnated filter.

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15. The method according to Claim 14 wherein said streptavidin-impregnated filter is positioned in a collection device.
- 5 16. The method according to any one of Claims 1 to 11 wherein said phosphoryl donor is ^{33}P - γ -ATP and wherein said step of monitoring or determining includes the use of scintillation proximity detection.
- 10 17. The method according to any one of Claims 1 to 11 wherein said step of monitoring or determining includes contacting said phosphorylated target substrate with antibody which selectively binds said phosphorylated target substrate to form a complex and detecting said
15 complex.
18. The method according to Claim 17 wherein said antibody is specific for a phosphohistidine residue in said phosphorylated target substrate.
- 20 19. The method according to Claim 17 wherein said antibody is specific for a phosphoaspartate residue in said phosphorylated target substrate.
- 25 20. The method according to Claim 17 wherein said step of detecting said complex includes detecting a detectable label attached to said antibody.
- 30 21. The method according to Claim 20 wherein said detectable label is selected from the group consisting of radionucleotides, dyes, enzymes, fluorescent compounds and biotin.

22. The method according to Claim 17 wherein said step of detecting is selected from the group consisting of latex agglutination, radioimmunoassay and ELISA.
- 5 23. A whole cell assay method of screening for inhibitors of fungal histidine kinase comprising the steps of:
- (a) growing a culture of fungal cells on a medium of low osmolarity lacking a putative inhibitor of said histidine kinase and determining a first growth
10 rate for said fungal cells;
 - (b) growing a culture of fungal cells on a medium of low osmolarity containing a putative inhibitor of said histidine kinase and determining a second growth rate for said fungal cells;
 - 15 (c) comparing said first growth rate and said second growth rate to determine if said putative inhibitor is an actual inhibitor of said histidine kinase.
24. A whole cell assay method of screening for inhibitors of
20 fungal histidine kinase comprising the steps of:
- (a) growing a first culture of fungal cells on a first medium of low osmolarity lacking a putative inhibitor of said histidine kinases;
 - (b) growing a second culture of fungal cells on a
25 second medium of low osmolarity wherein said second medium contains a putative inhibitor of said histidine kinases;
 - (c) waiting a known period of time to allow said first culture of fungal cells and said second culture of
30 fungal cells to grow;

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- (d) determining a first growth rate for said first culture of fungal cells and a second growth rate for said second culture of fungal cells; and
- (e) comparing said first growth rate with said second growth rate to determine whether said putative inhibitor is an actual inhibitor of said histidine kinases.
25. The method according to Claim 23 or Claim 24 wherein said fungal histidine kinase is a 2-component histidine kinase.
26. The method according to any one of Claims 23 to 25 wherein said fungal cells are selected from the group consisting of *Neurospora crassa*, *Candida albicans* and *Aspergillus fumigatus*.
27. A peptide useful as a substrate for histidine kinase assays selected from the group of amino acid sequences consisting of SEQ ID NOs: 3 to 12, a fragment thereof and an analogue thereof.
28. The peptide according to Claim 27 wherein said peptide has a chemical formula which includes said amino acid sequences and is smaller than 20 amino acids in length.
29. A kit for assaying histidine kinases, comprising:
- (a) a target substrate which can be phosphorylated in a reaction mixture containing histidine kinase and a phosphoryl donor thus yielding phosphorylated target substrate; and

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(b) means for detecting said phosphorylated target substrate.

5 30. The kit of Claim 29 wherein said target substrate is a biotinylated target substrate and wherein said phosphorylated target substrate is a biotinylated phosphorylated target substrate.

10 31. The kit according to Claim 29 or Claim 30 wherein said histidine kinase is a 2-component histidine kinase.

32. The kit of Claim 31 wherein said 2-component histidine kinase is a 2-component histidine kinase from a fungus.

15 33. The kit of Claim 32 wherein said fungus is selected from the group consisting of *Neurospora crassa*, *Candida albicans* and *Aspergillus fumigatus*.

20 34. The kit according to any one of Claims 29 to 33 wherein said target substrate includes a peptide.

25 35. The kit according to any one of Claims 29 to 34 wherein said target substrate comprises a peptide having at least one of an aspartic acid residue and a histidine residue.

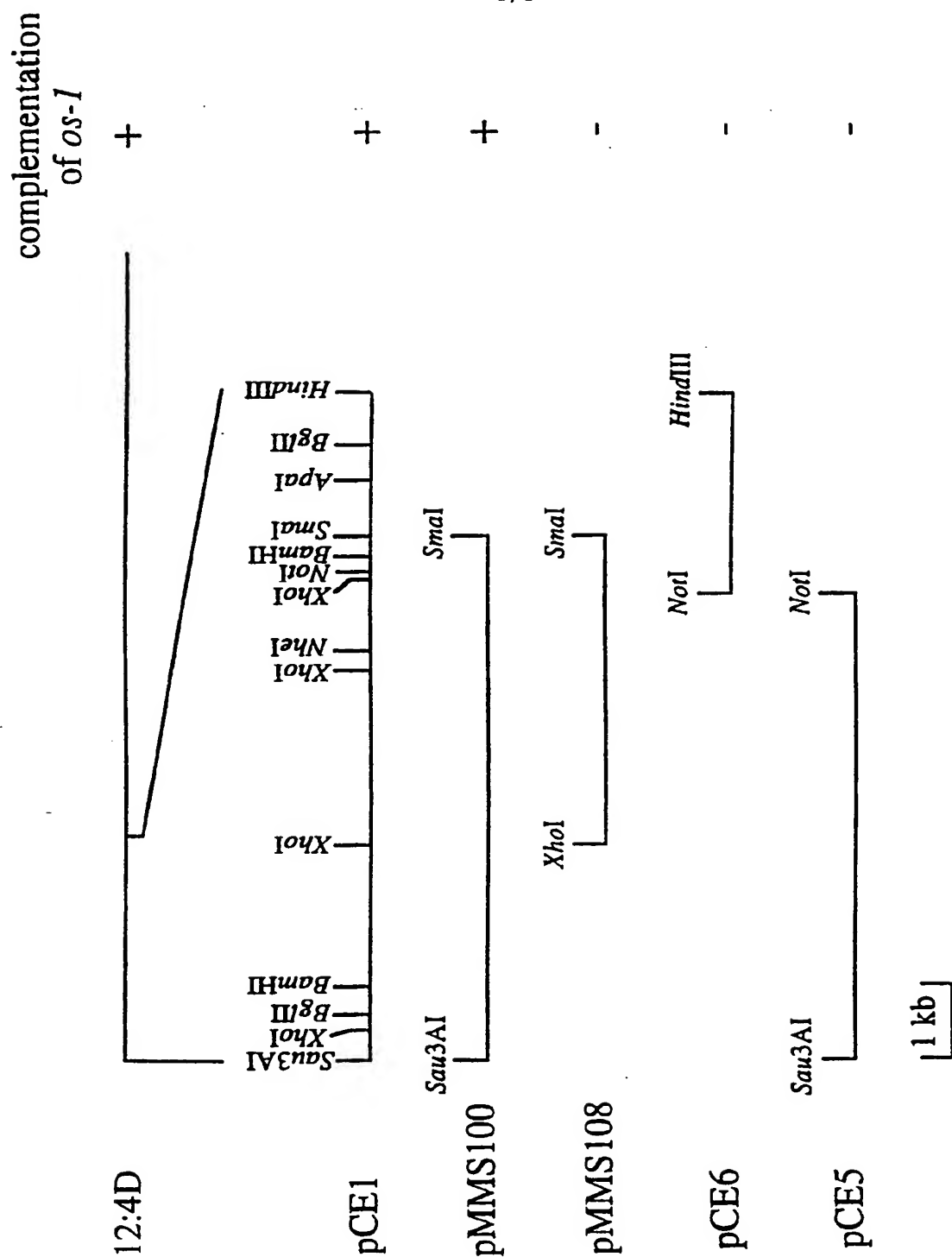
36. The kit according to Claim 35 wherein said peptide includes fewer than 20 amino acids.

30 37. The kit according to Claim 36 wherein said peptide comprises more than 10 amino acids.

38. The kit according to Claim 34 wherein said peptide is selected from the group of amino acid sequences consisting of SEQ ID NOs: 3 to 12, a fragment thereof, and an analogue thereof.
- 5 39. The kit according to Claim 29 wherein said means for detecting comprises a filter which binds said phosphorylated target substrate.
- 10 40. The kit according to Claim 39 wherein said filter is selected from the group consisting of phosphocellulose, nitrocellulose, nylon and nytran.
- 15 41. The kit according to Claim 29 wherein said detector means comprises a streptavidin-impregnated filter which binds said biotinylated phosphorylated target.
42. The kit according to Claim 29 wherein said means for detecting includes an antibody which selectively binds said phosphorylated target substrate to form a complex which is detectable.
- 20 43. The kit according to Claim 42 wherein said antibody is (1) specific for a phosphohistidine residue in said phosphorylated target substrate; or (2) specific for a phosphoaspartate residue in said phosphorylated target substrate.
- 25 44. The kit according to Claim 42 wherein said antibody contains a detectable label.
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45. The kit according to Claim 44 wherein said detectable label is selected from the group consisting of radionucleotides, dyes, enzymes, fluorescent compounds and biotin.



FIGURE

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 98/05746

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C1201/48 C1201/02

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C120 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 10461 A (PROMEGA CORP) 27 May 1993 see the whole document ---	1-22, 27-45
A	B S GOUELI, K HSIAO, A TEREBA, S A GOUELI: "A Novel and Simple Method to Assay the Activity of Individual Protein Kinases in a Crude Tissue Extract " ANALYTICAL BIOCHEMISTRY, vol. 225, 1995, pages 10-17, XP002071660 cited in the application see the whole document --- -/--	1-22, 27-45

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "&" document member of the same patent family

Date of the actual completion of the international search

15 July 1998

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/05746

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	Y F WEI, H R MATTHEWS: "A Filter-Based Protein Kinase Assay Selective for Alkali-Stable Protein Phosphorylation and Suitable for Acid-Labile Protein Phosphorylation" ANALYTICAL CHEMISTRY, vol. 190, no. 2, 1 November 1990, pages 188-192, XP002071662 see the whole document ---	1-22, 27-45
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P,A	WO 97 39143 A (ABBOTT LAB ;UNIV TECHNOLOGY CORP (US)) 23 October 1997 see the whole document -----	6,23-26, 32,33

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 98/05746

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			JP 7501444 T	16-02-1995
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